CHEMOKINE MEDIATED REGULATION OF TUMOR-LYMPHATIC CROSSTALK IN HEAD AND NECK CANCERS

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

Page

ABSTRACT 1

ACKNOWLEDGMENTS 2

CHAPTER

I. INTRODUCTION 3

II. METHODS 6

Cell Culture Techniques 6
Preparation of LEC-Conditioned Medium 6
RNA Isolation and RNA Quality Determination 7
cDNA Preparation 7
Real Time Polymerase Chain Reaction 7
Boyden Culturing, Staining and Imaging 7
miRNA In-Silico Analysis 8
Statistical Analysis 8

III. RESULTS 9

LECs Show Differential Migration Response to Different HNSCC Lines Indicating Possible Differences in Molecular Crosstalk 9
Several Cytokines and Chemokines Were Found to be Differentially Expressed in the Cancer Cells When Stimulated with LEC Conditioned Media 10
Several Genes that are Involved in Endothelial-to Mesenchymal Transition were Found to be Differentially Expressed in the Cancer Cells when Stimulated with LEC Conditioned Media 13
In Scilico Analysis 17

IV. CONCLUSION 18

REFERENCES 21

ABSTRACT

Chemokine Mediated Regulation of Tumor-lymphatic Crosstalk in Head and Neck Cancers

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A number of cancers disseminate first through the lymphatic system, and the presence of tumor cells in the sentinel or draining lymph node is a major prognostic indicator. Head and neck cancers (HNSCC) which preferentially migrate through lymphatics remain one of the most common cancers with an incidence of 600,000 new cases worldwide and a poor prognosis of 5 years survival. Yet mechanisms that contribute to HNSCC metastasis through lymphatics remain very poorly understood. Metastasis to lymph nodes is strongly correlated to morbidity, so further investigation into the mechanisms and factors that lead to tumor-lymphatic crosstalk and promote lymphatic metastasis is needed. The goal of my research is to characterize the cross talk between lymphatic endothelial cells (LECs) and tumor cells and analyze specific chemokines, cytokines, and inflammatory genes involved. In addition, we also want to evaluate the tole of EMT associated genes in this mechanism. Recently, miRNA have emerged as significant predictors of disease outcome and in silico analysis was carried out to determine their role in EMT associated pathways in this tumor lymphatic crosstalk. This research will be very valuable for the implementation of specific therapeutic strategies that are targeting tumors that metastasize through the lymph node and identify specific molecular pathways that could be targeted to arrest tumors at the site of entry.

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CHAPTER I

INTRODUCTION

Cancer is currently the second leading cause of death after heart disease. Of all cancer diagnosis it is believed that head and neck cancer diagnosis make up about 3% of diagnoses. Head and neck cancers are characterized by a primary tumor in the pharynx, larynx, nasopharynx, oropharynx, and sinuses [1]. It is estimated that in 2017, 79,970 patients were diagnosed with some form of head and neck cancer of which roughly 50% of the patients died [2]. Most patients diagnosed with head and neck cancers are diagnosed in later stages when their symptoms are more severe. When diagnosed in earlier stages patients can have a survival rate as high as 80%, however once the cancer becomes more aggressive locally or begins to metastasis the prognosis lowers to a less than 40% survival rate. When cancer metastases to a significantly distal site the chance of survival is extremely low [4-5]. Head and neck cancers can present with a variety of symptoms and are diagnosed with imaging and biopsies of tumors [3, 4]. As with most cancers tobacco use is a large causative agent of head and neck cancers. Other risk factors include age and sex. Men have been shown to have an increased risk of developing head and neck cancers, and that there is increased incidence with increasing age [2, 3, 5]. The largest sex disparities in cancers include esophageal and laryngeal cancers, where men are four times more likely to develop these cancers than women [2]. There are multiple treatments for cancer, and the best results are found from multiple paired therapies. Many head and neck cancers are treated surgically and with radiation therapy, but chemotherapy is also effective [1, 3, 4]. Currently there is a lot of research in the molecular mechanisms of head and neck cancer development and metastasis for further targeted therapies.

Metastasis is the largest prognostic factor for cancer patients. Metastasis occurs when tumor cells invade surrounding lymphatic or blood vasculature and migrate to a distal location. Once in a different location the cells must invade a surrounding tissue and begin to grow. Tumors can then promote lymphangiogenesis for further growth and metastasis[6]. For a cancer cell to proliferate and metastasize a cell must evade apoptotic factors and the body's immune system, and changes must occur that allow for invasion. Known factors that can influence a cell's ability to metastasis include inflammation, stress, recruitment of macrophages, hypoxic tumor environments, and epithelial-mesenchymal transition (EMT)[6]. While EMT is associated with healing, it is now known to be an integral part of metastasis[7, 8]. Tumor cells can preferentially metastasis through either the blood or lymphatic vasculature, and while much more research has been done with regards to blood vasculature metastasis, it is known that EMT is important in both for cell motility [6-8]. In metastasis tumor cells change phenotype after EMT to a mesenchymal phenotype to migrate through the blood stream. More research has been done recently into lymphatic metastasis which has a distinct disease process different from that of blood vasculature metastasis [9]. Prox1 and VEGFC play important regulatory roles in lymphatic cell migration and proliferation as opposed to blood endothelial cells [9, 10]. Learning more about lymphatic metastasis is important for targeted therapies that do not preferentially metastasis through the lymphatics like head and neck cancers.

Head and neck cancer with secondary metastasis is seen in about 15% of patients. Of the total patients with head and neck cancer in a single-patient analysis 15-20% of patients who died had metastatic cancer, and the median survival rate after being diagnosed with distally metastatic cancer was ± four months[11]. Extensive research has been done into changes in lymphatic endothelial cells that allow for tumoral development and in microenvironments that lead to

invasion for metastasis. Lymphangiogenesis and angiogenesis have been studied to discern what role they play in cancer metastasis. Through the use of Coculturing, PCR, Elisa, Western Blotting, Northern Blotting, Boyden Assays, and Seahorse XF Analysis other factors, or chemokines and cytokines, both have been determined to play an integral role in head and neck cancer metastasis. It is accepted that VEGF, LYVE1, EGFR, TGFB, P13K, MMP-9, CXCR1, and CXCR2 are all involved in the lymphatic metastatic pathway[12-17].

Also common in most tumors is microRNA dysregulation. MicroRNA are small RNA that have emerged as significant regulators of different diseases including cancers. miRNA inhibits gene expression by suppression of specific mRNA. Different miRNAs are involved in a regulatory compacity in pathways important for tumor proliferation, invasion, and migration[13, 18]. Research on the role microRNA plays in cancer development and metastasis has increased as it has become increasingly clear that it has tremendous potential for therapeutic targeting.

This thesis will explore mechanisms by which head and neck cancers preferentially metastasize through the lymphatic vasculature. The mechanism of metastasis will be investigated by looking at various components that are known to contribute to the process of invasion and dissemination including inflammation, chemokines and cytokines, and the role of Endothelial Mesenchymal Transition (EMT). The goal of my research is to characterize the cross talk between lymphatic endothelial cells and tumor cells that takes place for metastasis to occur. Recently miRNA have emerged as significant predictors of disease outcome and we have identified by in silico analysis several miRNAs that target the specific pathways and chemokines or cytokines shown to be dysregulated in this tumor lymphatic crosstalk. Through in silico analysis the miRNA that modulate these processes will also be explored.

CHAPTER II

METHODS

Cell Culture Techniques

Lymphatic endothelial cells (LECs) were cultured in Endothelial Cell Growth Medium (EGM) MV2 with 1% Penicillin/Streptomycin and 10% FBS. LECs were passaged every 72 hours using HEPES BSS, EDTA, and TNS. FaDu, Detroit 562, and SCC9 cell lines were obtained from the American Tissue Culture Collection. SCC9 was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with 100 mM of sodium pyruvate, 400 ng/uL hydrocortisone, 10% FBS, and 1% Antibiotic-Antimycotic. FaDU and Detroit 652 were cultured in Minimum Essential Medium Eagle's with 10% FBS, and 1% Antibiotic-Antimycotic. All cancer cell lines were passaged with DPBS, without calcium or magnesium, and .25% EDTA. All cells were maintained, and passaged separately and incubated in separate incubators both at 37 C with 5% CO_2.

Preparation of LEC-Conditioned Medium

HDLEC conditioned medium (LCM) was prepared by incubating the cells in 5% FBS containing EGM MV2 (without supplement mix) for 48h. the medium was then removed, centrifuged (1000g, 8 min) and stored frozen at -80°C prior to use. SCC9 conditioned medium (TCM) was prepared by incubating the cells with plain EGM MV2 medium for 48h. The medium was collected, centrifuged and filter sterilized and stored frozen at -80°C. 5% FBS was added to this prior to use.

RNA Isolation and RNA Quality Determination

RNA was isolated from the cell lines following the manufacturer's instructions using the RNAeasy Mini Kit (QIAGEN, Germantown, MD). RNA was then quantified using a NanoDrop, Quality of RNA was determined by measuring absorbance at 260/280.

cDNA Preparation

1ug of RNA was converted to cDNA by the manufacture instructions using Maxima H Minus cDNA Synthesis Master Mix (Thermo Scientific, Waltham, MA). The final cDNA was diluted to 5ng/µl.

Real-Time Polymerase Chain Reaction

Gene expression was determined by Real-Time Polymerase Chain Reaction (PCR). PCR was carried out by SYBR Green Real Time PCR Master Mix (Thermo Scientific, Walthman, MA). All primers were purchased from Sigma Aldrich (St. Louis, MO). Ubiquitin was used as the housekeeping gene for normalization in analysis of gene expression. The forward primer for ubiquitin was 5' AGTCCCTTCTCGGCGATTCT 3' and the reverse primer was 5' GCATTGTCAAGTGACGATCACAGC 3'. PCR data was analyzed using a two-way ANOVA on GraphPad Prism 7.

Boyden Culturing, Staining and Imaging

Cancer cell lines were cultured in a 12-well plate in EGM with 5% FBS. Once these cells lines are confluent, 8µM standing Boyden chamber inserts (coated with collagen-50µg/mL) are placed in each well and $1X10^5$ cells lymphatic endothelial cells are put in each insert. After 48, the inserts were taken out, washed, cells in the bottom were fixed with ice cold methanol and stained with 0.5% crystal violet and images were taken using an inverted microscope. The number of cells were counted using Image J software..

miRNA In-Silico Analysis

In Silico analysis was performed using miRwalk database version 3.0 (http://mirwalk.umm.uni-heidelberg.de). miRNA that target the EMT genes was analyzed to determine common miRNA and pathways that might be activated by the genes.

Statistical Analysis

Data were analyzed with Graph pad prism software using ANOVA followed by Fisher LSD or using t-test. Mean values between two groups were compared using the Student's unpaired t-test and statistical significance was defined as $P < 0.05$.

CHAPTER III

RESULTS

LECs Show Differential Migration Response to Different HNSCC Lines Indicating Possible Differences in Molecular Crosstalk

It is accepted that there is an attraction between tumor cells and the lymphatic vessel to promote tumor cell invasion. Previous data from the lab shows that SCC9 migrate in response to LECs and that this migration varies with inflammation. To look at the opposite relationship LEC migration in response to the different cancer cell lines. Representative mages take to show the different migration of LECs is shown in Figure 1. The migration in response to the different cell lines were compared to control migration and shown in Figure 2.

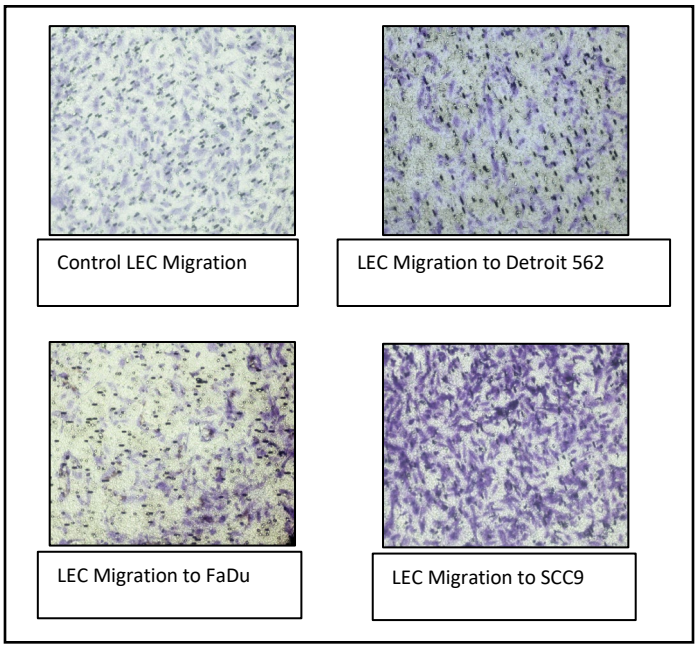

Figure 1. LEC migration in response to different HNSCC lines. Representative images of migrated cells (10X). SCC9 cells were grown in 12 well plate and $1X10^5$ LECs were counted and

put in 0.8micron inserts and allowed to migrate for 24 hours and were pictured using 10X. Number of cells migrated was counted using ImageJ software.

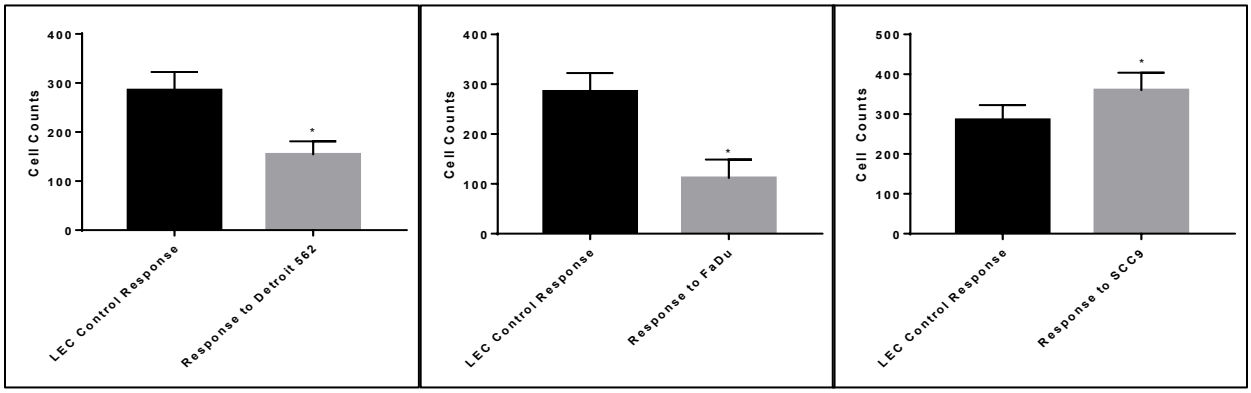

Figure 2. Cells that migrated towards the various cancer cells were quantified and plotted. Values represent mean ± SEM. *, represent values significantly different $p \leq 0.05$ when compared control

Several Cytokines and Chemokines were Found to be Differentially Expressed in the Cancer Cells when Stimulated with LEC Conditioned Media

Several chemokine and cytokines are known to participate in the cross talk between cancer cells and lymphatic vessels. However, the detailed mechanisms or the specific chemokines and cytokines that mediate thes interactions are not completely understood. To investigate what these chemokines and cytokines real time PCR was carried out to assess gene expression level in the different cancer cell lines when treated with LEC conditioned media as compared to control. The different expression of the chemokines or cytokines tested are shown in Figure 3, Figure 4, and Figure 5.

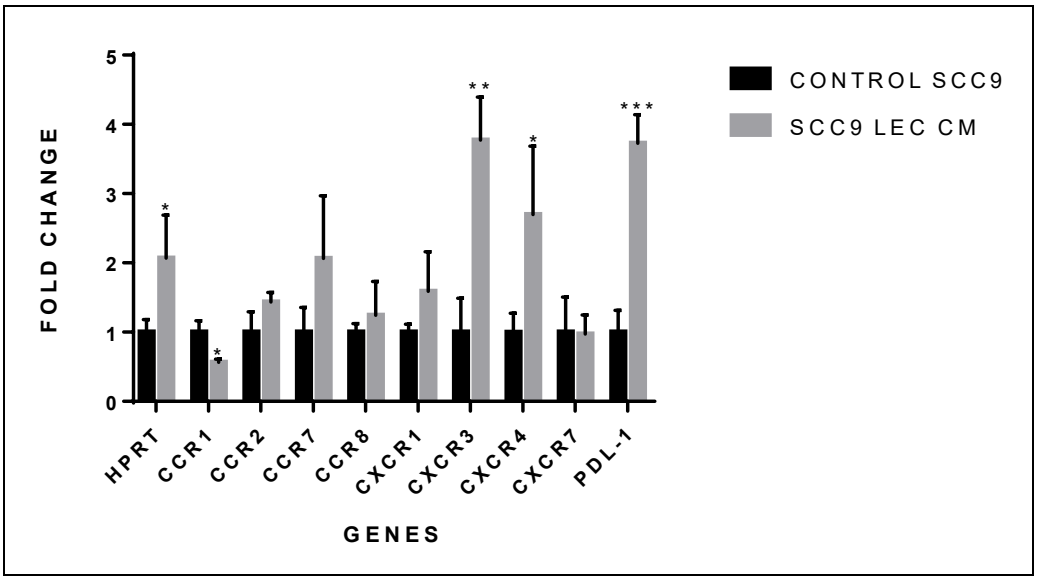

Figure 3. Differential expression of cytokines and chemokines in SCC-9 cells in response to LEC-Conditioned Medium. The mRNA levels of chemokines in LCM induced SCC9 cells were quantified by real time PCR. Expression of HPRT, CCR1, CCR2, CCR7, CCR8, CXCR1, CXCR3, CXCR4, CXCR7 and PDL-1 were quantified and plotted. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. *, represent values significantly different $p \leq 0.05$ when compared control

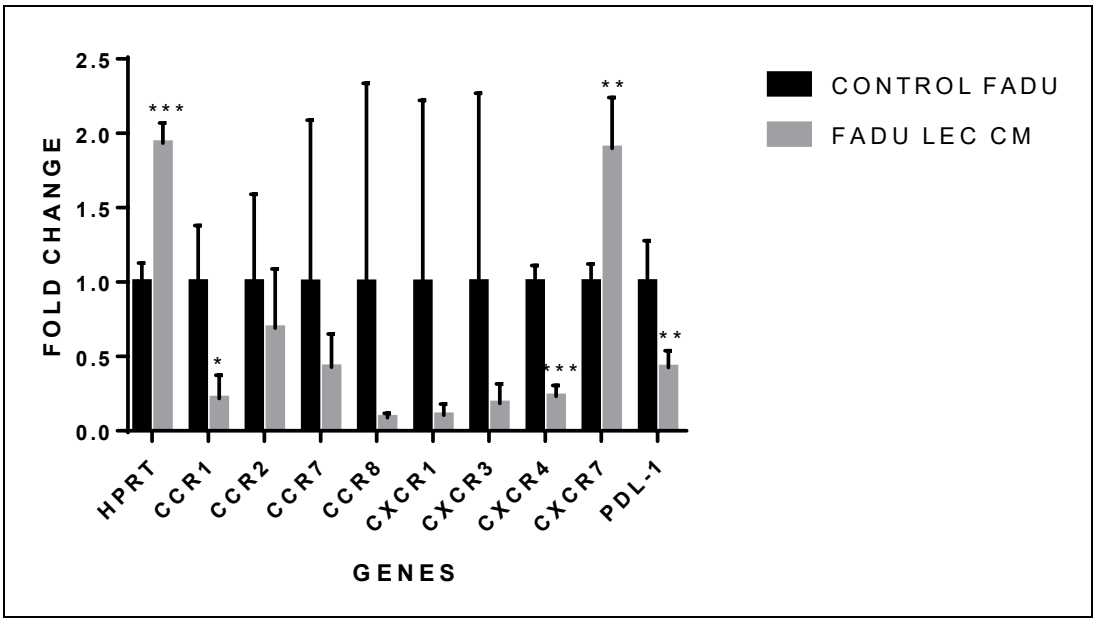

Figure 4. Differential expression of cytokines and chemokines in FADU cells in response to LEC-Conditioned Medium. The mRNA levels of chemokines in LCM induced FADU cells were quantified by real time PCR. Expression of HPRT, CCR1, CCR2, CCR7, CCR8, CXCR1, CXCR3, CXCR4, CXCR7 and PDL-1 were quantified and plotted as above. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. *, represent values significantly different $p \leq 0.05$ when compared control.

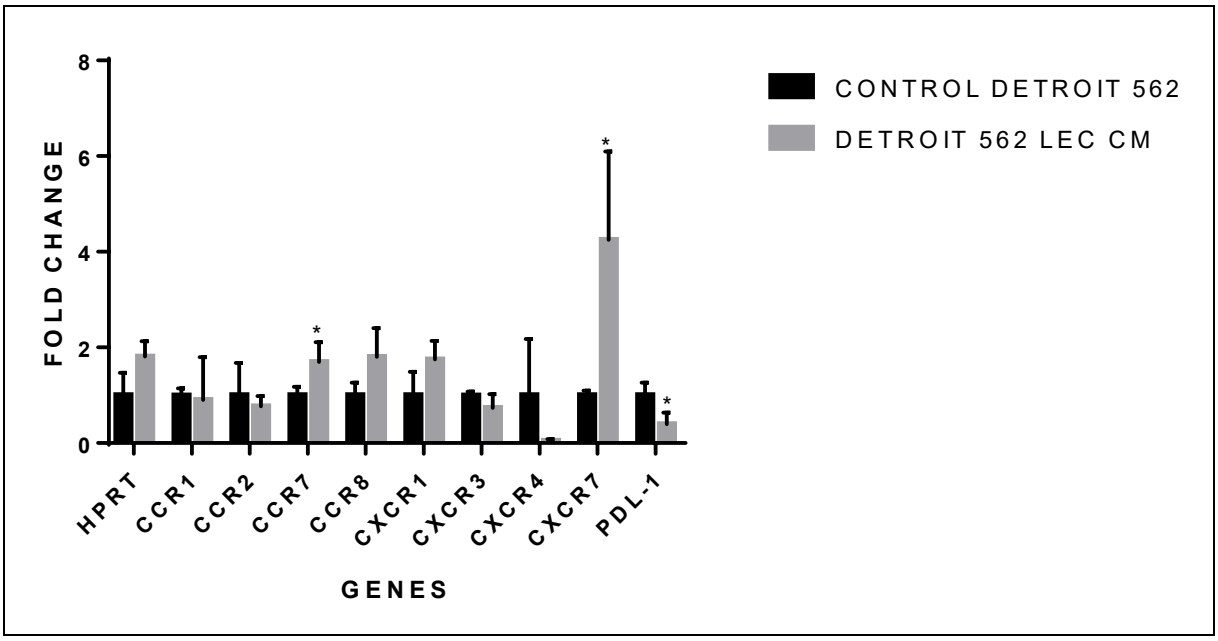

Figure 5. Differential expression of cytokines and chemokines in FADU cells in response to LEC-Conditioned Medium. The mRNA levels of chemokines in LCM induced FADU cells were quantified by real time PCR. Expression of HPRT, CCR1, CCR2, CCR7, CCR8, CXCR1, CXCR3, CXCR4, CXCR7 and PDL-1 were quantified and plotted. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. *, represent values significantly different $p \leq 0.05$ when compared control.

Several Genes that are Involved in Endothelial-to Mesenchymal Transition were Found to be Differentially Expressed in the Cancer Cells when Stimulated with LEC Conditioned Media

EMT is a transition of epithelial cells a mesenchymal phenotype resulting in polarity changes. With the mesenchymal phenotype cells lose their ability to form tight cell junctions promoting cancer cell invasion and later dissemination. It is thought that EMT plays a large role in lymphatic metastasis so it is expected the EMT related genes will be dysregulated in cancer cells in response to cytokines and chemokines produced by LECs. The gene expression of EMT

associated genes in cancer cell lines treated with LEC CM as compared to control is shown in Figure 6, Figure 7, and Figure 8.

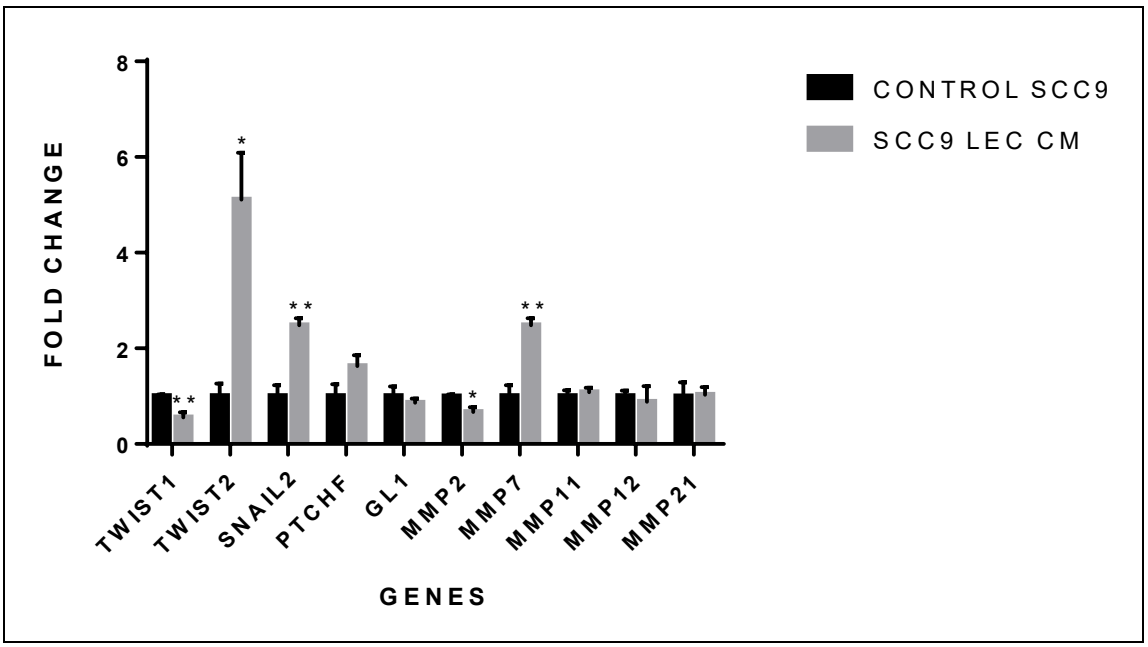

Figure 6. Several EMT associated genes were found to be differentially regulated in SCC-9 cells in response to LEC-Conditioned Medium. The mRNA levels of EMT genes in LCM induced SCC-9 cells were quantified by real time PCR. Expression of TWIST1, TWIST2, SNAIL2, PTCHF, GLI, MMP2, MMP7, MMP11, MMP12, MMP21 were quantified and plotted. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. **, represent values significantly different $p \leq 0.05$ when compared control.

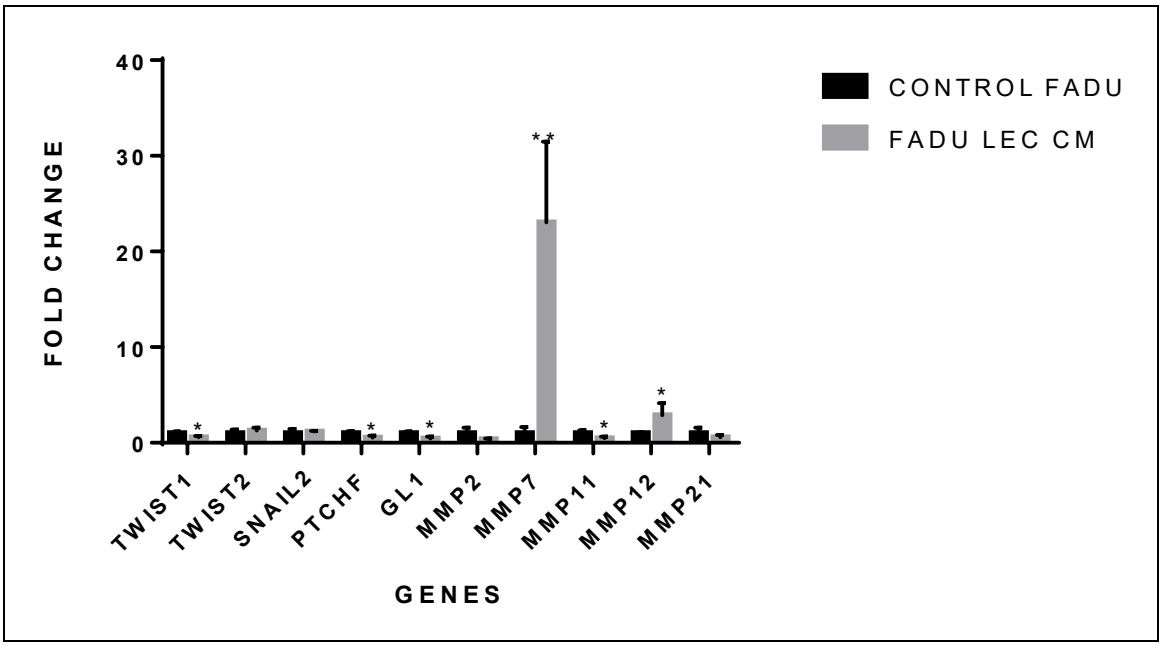

Figure 7. Several EMT associated genes were found to be differentially regulated in FADU cells in response to LEC-Conditioned Medium. The mRNA levels of EMT genes in LCM induced FADU cells were quantified by real time PCR. Expression of TWIST1, TWIST2, SNAIL2, PTCHF, GLI, MMP2, MMP7, MMP11, MMP12, MMP21 were quantified and plotted. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. **, represent values significantly different $p \leq 0.05$ when compared control.

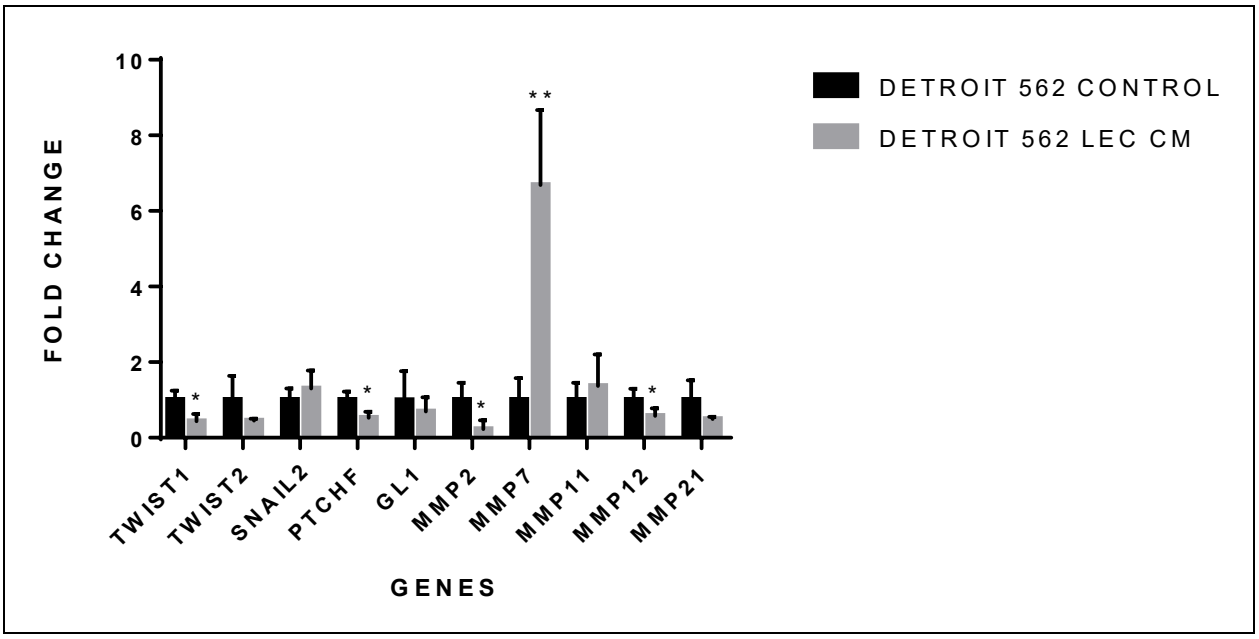

Figure 8. Several EMT associated genes were found to be differentially regulated in Detroit cells in response to LEC-Conditioned Medium. The mRNA levels of EMT genes in LCM induced Detroit cells were quantified by real time PCR. Expression of TWIST1, TWIST2, SNAIL2, PTCHF, GLI, MMP2, MMP7, MMP11, MMP12, MMP21 were quantified and plotted. Ubiquitin was used as a housekeeping control. Values represent mean ± SEM. *, represent values significantly different $p \leq 0.05$ when compared control.

In Scilico Analysis

miRNA are small, 18-22 nucleotide long strands of RNA that regulate gene expression. miRNA are known to be predictors of disease outcome and can be easily target by overexpression or inhibition. To see what miRNA that regulate EMT processes during cancer-lymphatic crosstalk in silico analysis was performed on genes of significance. Figure 9 is a map that shows the relationship found.

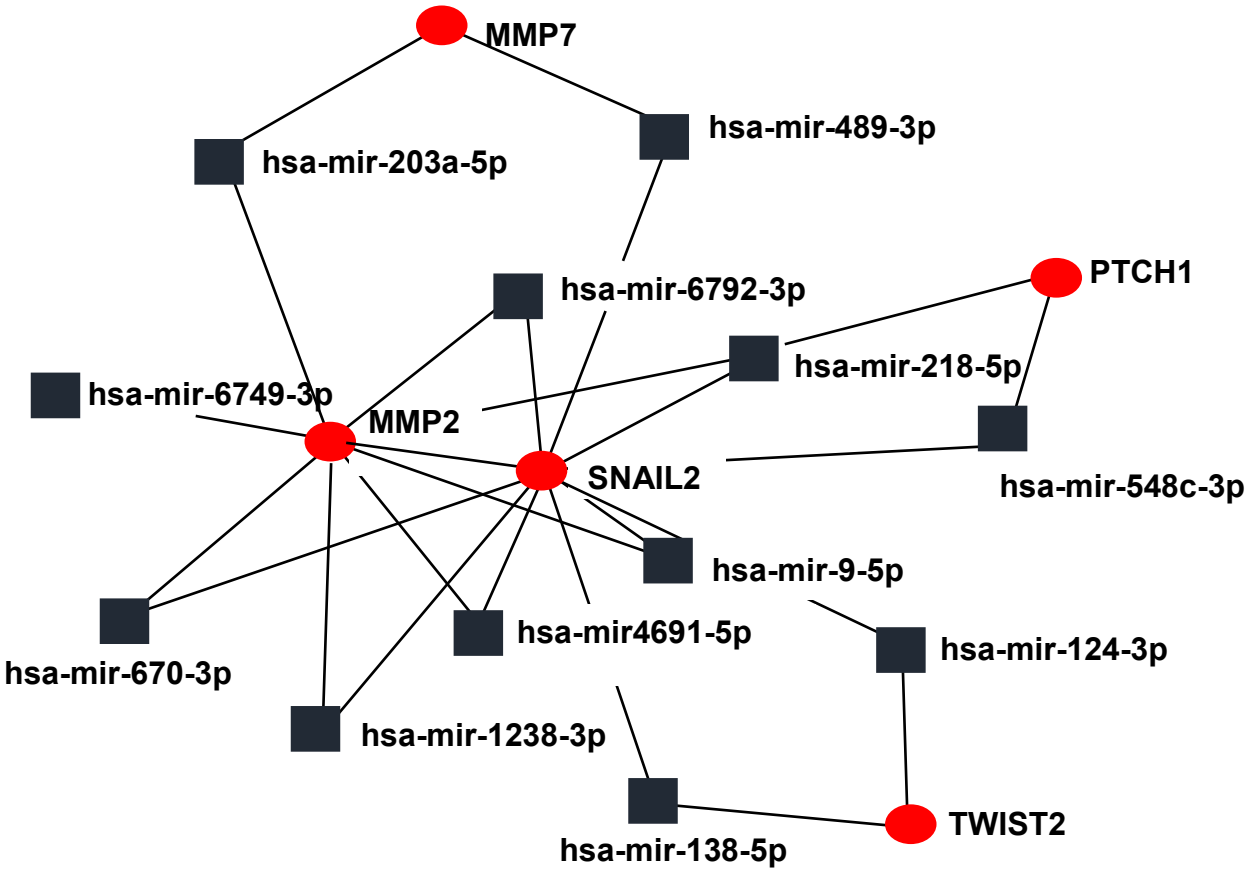

Figure 9. In-Silico analysis of EMT genes associated with lymphatic metastasis shows association with several miRNA involved in lymphatic inflammation or lymphangiogenesis.

CHAPTER IV

CONCLUSION

It is evident that specific molecular pathways in the tumor microenvironment potentially predispose tumors to enter lymphatics. In response to SCC9 cells LEC cells migrated significantly ($p<.05$) when compared to control. LEC migration however did not increase in response to FaDu and Detroit 562 cell lines ($p<0.05$). This shows that LECs show increased invasion in response to head and neck cancer cells but this effect is differential and likely depends on the specific cytokine and chemokine mechansisms and molecular crosstalk between the tumor cells and the lymphatic endothelium.

SCC9, FaDu, and Detroit 562 cells treated with LEC conditioned medium expressed different levels of cytokines, chemokines, and other molecules when compared to control. One gene that showed significant difference in all three cell lines is PDl-1. PDL-1 is thought to be involved in cancer cell immune evasion[19]. PDL-1 was increased significantly in SCC9, and was significantly decreased in FaDu and Detroit 562 cell lines. Increased PDL-1 expression has been shown in oral squamous cell and in non-small cell lung cancers. CXCR3 and CXCR4 are significantly increased in SCC9 cells which are chemokines involved in the process of inflammation. CXCR3 is involved in the recruitment of activated T-cells, and CXCR4 is associated with tumor growth [20, 21]. CXCR4 expression is decreased FaDU cells in response to LEC CM. CXCR7 expression is increased in both FaDu and Detroit 562. CXCR7 is a associated with tumor prognosis and is increased in various tumors. CCR1 is another chemokine associated with the process of inflammation which is decreased in expression in SCC9 and FaDu cell lines. CCR7 is involved in the control of the migration of immune cells and was increased in Detroit 562. Finally,

HPRT expression is increased in SCC9 and FaDu. HPRT is induced by HIF1a which is associated with hypoxia. Hypoxic tumor environments are associated with increased cancer cell invasion and dissemination. These results demonstrate the cell lines treated with LEC conditioned media express different levels of cytokines, chemokines, and other molecules that assist in cancer metastasis.

Several EMT associated genes are found to be dysregulated when the different cancer cell lines are treated with conditioned media. TWIST 1 and MMP7 are significantly differentially expressed when compared to control in all cell lines. TWIST 1 is considered a regulator of EMT because it is involved in the downregulation of E-cadherin. TWIST 1 is decreased in all three cell lines. MMP7 is a protein involved in breakdown of the extracellular matrix, that is increased in all three cells lines which may be indicative of increased invasion properties of these cell lines in response to LECs. TWIST 2 and SNAIL2 are both significantly increased in SSC9 cells. These are both considered EMT regulators like TWIST1 for similar role in E-cadherin expression [22]. MMP2, like MMP7, are is involved in extracellular matrix remodeling and is increased in SCC9 and in Detroit 562. Also included in the MMP family is MMP12 which is increased in FaDu cells and decreased in Detroit 562 cells, and MMP11 which is significantly decreased in FaDu cells. In EMT the MMP family is typically increased to promote invasion [22]. PTCHF was significantly decreased in both FaDu and Detroit 562. PTCHF is a known tumor suppressor.

In-Silico analysis of the EMT genes that showed differential regulation across multiple cancer cell lines such at Patched, TWIST2, SNAIL2, MMP2 and MMP7 were analyzed using miRWalk and TargetScan to determine common interacting miRNAs. Our analyses showed the interaction with several miRNAs that are known to be involved in EMT or cancer initiating and promoting pathways. In particular one miRNA that was found to be associated with several EMT

genes was miR-9. This is interesting as earlier miR-9 was found to be involved in both suppression of inflammation and activation of lymphangiogenic pathways in the LECs [23]. Several other interesting miRNAs were also found that warrant more detailed analysis on their functional effects and specific target repression in the LECs or the cancer cells.

Future plans from this research include delineating specific chemokines and cytokines that mediate the tumor-lymphatic cross talk, profiling miRNA that are differently expressed in LECs cocultured with the different cell lines, and over-expressing or inhibiting one of the most differentially expressed miRNA to analyze their function.

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